AuPS/ASB Meeting - Adelaide 2010

Free communications: Ion channels

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Chair: Peter Barry

Agonist interactions and selectivity in $GABA_{A/C}$ receptors

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Cys-loop ligand-gated ion channels constitute one of two major superfamilies of receptors mediating rapid chemical synaptic transmission in the central nervous system. They include cation selective channels that are receptors for excitatory neurotransmitters, acetylcholine and serotonin, and anion selective channels that are receptors for inhibitory neurotransmitters, γ-aminobutyric acid (GABA) and glycine. Recent structural information from snail acetylcholine binding proteins (AChBP), torpedo acetylcholine receptors and bacterial homologs have provided a good understanding of the overall structure of the superfamily and of specific details of acetycholine-receptor interactions. For inhibitory receptors for GABA and glycine, however, we have a much more limited understanding of how receptors interact with and are selectively activated by particular agonists.

In this study, we have investigated interactions between GABA and receptor, using the homopentameric ρ1 γ-aminobutyric acid receptor ($GABA_C$) as a model for the broader family of heteropentameric $GABA_A$ receptors. We used homology modeling to identify a series of conserved charged residues at the GABA-binding site that we hypothesized formed a series of charge-charge interactions likely to be important for interaction with agonist, agonist selectivity and receptor activation. We have tested this hypothesis using site-directed mutagenesis in combination with two-electrode voltage clamp recording of recombinant receptors expressed in *Xenopus* oocytes. Preliminary results have revealed key determinants of agonist selectivity, particularly determining sensitivity to the size or length of the ligand, as well as receptor activation or gating. These results are consistent with our hypothesis and provide a basis for a more detailed understanding of agonist-receptor interactions in inhibitory Cys-loop ligand-gated ion channels.

Defining $GABA_A$ receptor pharmacology and physiologies through the disruption of receptor protein interactions

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$GABA_A$ receptors are the dominant inhibitory neurotransmitter-gated ion channel in the central nervous system. We have identified a novel way in which these neuronal ion channels alter their electrical response. Interactions between neighbouring, clustered $GABA_A$ receptors profoundly alter single-channel properties (conductance and kinetics), leading to a significant enhancement of channel activity ('cross-talk') (Everitt *et al.*, 2009). Interactions were identified using competitor peptides that mimic defined intracellular protein binding sites. Peptides were applied directly onto inside-out membrane patches pulled from newborn rat hippocampal neurons and single-channel currents were recorded. Combining the use of competitor peptides and single-channel recordings provided a visual insight into the dynamic nature of protein interactions that affect the activity of single $GABA_A$ ion channels. Specifically, when applied to inside-out patches, a peptide mimicking the MA helix of the γ2 subunit (γ381-403) of the $GABA_A$ receptor abrogated the potentiating effect of the drug diazepam on endogenous receptors by substantially reducing their conductance.

In addition to benzodiazepines, barbiturates, general anaesthetics and neurosteroids have all been shown to facilitate neuronal receptor cross-talk, that is, the drugs potentiate GABA-activated currents increasing both channel open probability and conductance. Such drugs however, are predicted to act on different $GABA_A$ receptor subtypes. We hypothesized therefore, that modulation of ion permeation was a general mechanism through which all $GABA_A$ receptor subtypes signal. Using a competitor peptide specific to δ-containing $GABA_A$ receptors we tested our hypothesis. GABA currents were potentiated by the general anesthetic etomidate and competitor peptides were applied to neuronal patches. Addition of the δ MA peptide but not a scrambled version or the γ MA peptide abrogated the potentiating effects of etomidate. These data support our hypothesis and are aiding our understanding of the complex interplay between drugs and ion channels and also amongst the different $GABA_A$ receptors subtypes themselves.

Everitt AB, Seymour VA, Curmi J, Laver DR, Gage PW, Tierney ML. (2009) Protein interactions involving the γ2 large cytoplasmic loop of $GABA_A$ receptors modulate conductance. *FASEB Journal* **23:** 4361-4369.

Developing activation mechanisms for $GABA_A$ receptors

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The α1β2γ2 and α3β3γ2 are two synaptic isoforms of α-aminobutyric acid type A ($GABA_A$) receptor. They are found at different synapses, for example in the thalamus, where they mediate different inhibitory postsynaptic current profiles, particularly with respect to the rate of current decay. The kinetic characteristics of both isoforms were investigated by analysing single-channel currents over a wide range of GABA concentrations. α1β2γ2 channels exhibited briefer active periods than α3β3γ2 channels over the entire range of agonist concentrations and had lower intraburst open probabilities at subsaturating concentrations. Activation mechanisms were constructed by fitting postulated reaction schemes to data recorded at saturating and subsaturating GABA concentrations, simultaneously. Reaction mechanisms were ranked according to goodness of fit values to open and shut dwell histograms of single channel activity, and how accurately they simulated ensemble currents. The highest ranked mechanism for both channels consisted of two sequential binding steps, followed by three conducting and three nonconducting configurations. The equilibrium dissociation constant for GABA at α3β3γ2 channels was ~3 μM compared with ~19 μM for α1β2γ2 channels, suggesting that GABA binds to the α3β3γ2 channels with higher affinity. A notable feature of the mechanism was that two consecutive doubly liganded shut states preceded all three open configurations. The lifetime of the third shut state was briefer for the α3β3γ2 channels. The longer active periods, higher affinity, and preference for conducting states are consistent with the slower decay of inhibitory currents at synapses that contain α3β3γ2 channels. The reaction mechanism we describe accurately simulates real macropatch and synaptic currents mediated by the two $GABA_A$ receptor subtypes and may be appropriate for the analysis of other $GABA_A$ receptor isoforms. The mechanism may also be applicable for the rational investigation of the kinetic effects of therapeutic agents that activate and modulate $GABA_A$ receptors, in addition to mutated channels that give rise to disease.

Understanding the molecular and pharmacological basis of selectivity of nicotinic acetylcholine receptor antagonists using reactive methyllycaconitine analogues

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The nicotinic acetylcholine recepto (nAChR) mediates fast synaptic transmission between neural cells. The nAChR is a pentameric protein that contains a large extracellular domain, four transmembrane domains (M1-M4) where the second M2 lines the channel pore, two short M1-M2 and M2-M3 loops that move to gate the channel and a large intracellular M3-M4 loop. There is a large amount of subunit heterogeneity within the nAChR, which can be formed by specific combinations of α2-10 and β2-4 receptors. The expression patterns of receptor subtypes partly determine the physiological role of each nAChR subtype. Thus, pharmacological agents that can distinguish between receptor subtypes may have greater selectivity for certain physiological process, and may provide superior pharmacological agents. The α7 homomeric is potently and selectivity inhibited by the toxin methyllcaconotine (MLA) from the lakspur plant. Our aim was to identify the site of the receptor that conferred the binding selectivity to MLA on the α7 receptor and compare this to the corresponding residues on the α4β2 receptor. The α7, α4 or β2 cRNA was injected into *Xenopus* oocytes that were removed from frogs anaesthetized with tricaine and ion channel function was measured by the two-electrode voltage clamp technique. For efficient expression of the α7 nAChR, cRNA for the chaperone protein RIC-3 was co-injected. To prevent the large desensitization properties of the α7 nAChR, a mutant L9′T DNA was created by site directed mutagenesis and all further mutations were studied with this background. The L9′T mutation markedly affected acetylcholine activation but not MLA sensitivity. When varying concentrations of ACh were applied to oocytes injected with α7 or α7L9′T after 3 minute incubation with a set concentration of MLA, the maximum response was the same as for the maximum response to ACh alone. This suggests that the ACh is competing for the same binding site with the MLA. Furthermore, the IC50 of MLA is significantly reduced in the α4β2 nAChRs, highlighting the selectivity. When this experiment was performed on oocytes injected with α4β2 nAChRs, the ACh the maximum response with ACh and MLA was significantly lower than ACh alone, indicating that the MLA was also binding at a site different to the ACh-binding site. A previous published crystal structure of the acetylcholine binding protein bound to MLA identified residues that interact directly with the MLA molecule. We focused on two sites where MLA was bound, including the Q79 residue where several antagonists and agonists of the α7 nAChR confer selectivity by interactions with this residue in the extracellular domain. We have mutated this residue to the lysine and threonine residues that are the homologous residues on the α4 and β2 receptors, respectively to create the Q79K L9′T and Q79T L9′T mutant receptors. We have also made the homologous reversal mutations on the α4 and β2 subunits to determine if the MLA inhibition is altered. A second approach was taken by modifying MLA to contain a cysteine-reactive MLA molecule that can tether to introduced cysteines on the target receptor. We applied this molecule to α7 receptors with introduced cysteine residues and identified one residue, S188C L9′T, where the addition of the cysteine reactive MLA causes a permanent reduction in the current elicited by ACh. This indicates a strong association between this residue and the site of the cysteine reactive group in MLA binding. We have made the corresponding mutations in the α4 and β2 subunits to compare the residues that bind to MLA in nAChR subtypes. Here we show that the while the residues that bind to selective antagonists of nAChRs can be predicted with homology models, the mechanism by which these antagonists are selective are best understood by studies using a combination of site-directed mutagenesis and chemical modification.

Reciprocal regulation of expression of STIM1 and Orai1 proteins

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Two proteins, stromal interaction molecule 1 (STIM1) and Orai1 constitute the minimum molecular components of the Ca^{2+} release-activated Ca^{2+} (CRAC) channel (Liou *et al.*, 2005; Roos, *et al.*, 2005; Vig, *et al*., 2006). STIM1 is predominantly located in the membrane of the endoplasmic reticulum (ER) and functions as a molecular sensor of free ER Ca^{2+}, whereas Orai1 is located on the plasma membrane and when activated by STIM1 forms the Ca^{2+} selective pore of the channel (Yeromin *et al.*, 2006). While activation of CRAC channels uniquely depends on the free Ca^{2+} concentration in the ER lumen, its inactivation is regulated by both the free ER [Ca^{2+}] and the cytosolic [Ca^{2+}]. Fast Ca^{2+}-depend inactivation (FCDI) is a feedback mechanisms which limits Ca^{2+} entry through these channels at negative potentials and is regulated by Ca^{2+} binding to surface composed of residues from both Orai1 and STIM1 (Mullins *et al*., 2009; Lee *et al.*, 2009). Previously we identified that FCDI of I_{CRAC} depends on the relative expression levels of the STIM1 and Orai1 proteins (Scrimgeour *et al.*, 2009). Herein we present data that suggests the presence of another Ca^{2+}-dependent mechanism which regulates the activity of CRAC channels. Specifically, the expression of STIM1 and Orai1 are interdependent and also [Ca^{2+}]-dependent.

Heterologous expression of STIM1 and Orai1 was conducted in HEK293T cells using the plasmid/DNA vectors pEX-GFP-Myc-Orai1, pCMV-Sport6-STIM1, pCMV-Sport6-Orai1, Sport6-Orai1Δ70-88 and pCIneo-hClC-1 which were co-transfected at different ratios (between 1:8 and 8:1 of Orai1:STIM1) using PolyFect transfection reagent (Qiagen). The relative expression of STIM1 and Orai1-GFP proteins was determined using quantitative western blot analysis using anti-STIM1 and anti-GFP antibodies. GAPDH was used as an internal loading control.

Increasing the amount of Orai1 containing plasmid in the transfection mixture resulted in a significant decrease in STIM1 expression. In contrast, control experiments using expression of either, non-functional Orai1Δ70-88 or the unrelated ClC-1 protein had no effect on the expression levels of STIM1, identifying that the Orai1-STIM1 interaction was not a non-specific effect of competition in co-transfection. Depletion of intracellular Ca^{2+} stores, using thapsigargin, which activates Ca^{2+} entry through CRAC channels, increased the dependence of STIM1 expression on the Orai1. In contrast, inhibition of Ca^{2+} entry by 2-aminoethoxy-diphenyl borate (2-APB) or La^{3+} virtually abolished the interdependence of STIM1 and Orai1 expression.

These data indicate that the expression of STIM1 and Orai1 proteins is interdependent and is regulated in a Ca^{2+}-dependent manner which may provide an important cellular feedback mechanism to enable medium to long term regulation of ER Ca^{2+} homeostasis.

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